Immobilization of a phosphonium ionic liquid on a silica monolith for hydrophilic interaction chromatography

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Abstract

A methodology for preparing phosphonium-based ionic liquid modified silica-based monolithic capillary columns is presented. The columns with dimensions of 150 x 0.1 mm were modified by a phosphonium-based ionic liquid (trioctyl(3/4-vinylbenzyl)phosphonium chloride) *via* 3-(trimethoxysilyl)propyl methacrylate. The prepared columns were evaluated under hydrophilic interaction liquid chromatography separation conditions, employing a sample mixture containing purine and pyrimidine bases and nucleosides. Detection was made by UV. The high efficiency of the original silica monolith was preserved even after modification, and it reached values in the range of 98 000 - 174 000 theoretical plates/m. The effects of the concentration of acetonitrile in the mobile phase, the presence of additives in the mobile phase, such as, acetic acid or ammonium acetate, and the pH of the mobile phase on

the separation of some selected analytes were investigated. The prepared columns showed different separation selectivity compared to silica, phenyl and sulfobetaine stationary phases.

1 Introduction

Research on new particulate sorbents, as well as monoliths, continues in laboratories worldwide [1-5]. New classes of chemistries are accompanied with synthesis, especially for the preparation of monoliths, where new synthesis routes or monomers are employed. The desired monolithic stationary phases can be prepared utilizing one-step or multiple-modification preparation procedures. The first approach covers the preparation of monoliths, where all components including monomers with appropriate functional groups are present in the starting mixture. This approach will result in the direct formation of a functionalized monolith. The latter approach involves gradual modification of the original monolith to obtain the desired stationary phase. A detailed overview of the preparation of inorganic, polymer, or hybrid monolithic columns and their utilization in chromatographic separations can be found in many reviews, see, *e.g.*, [2, 3, 6, 7] for some recent works dealing with the topic.

Phosphonium-based ionic liquids are used in industry mainly as extraction solvents, chemical synthesis solvents, electrolytes in batteries, and as super-capacitors. An overview of their applications in these fields is found, *e.g.*, in a review by Fraser and MacFarlane [8]. Successful attempt to utilize polymerized phosphonium ionic liquids as stationary phases in gas chromatography has been also presented [9]. Last year, Dier and co-workers presented gradient separation of complex sample mixtures, originating from electrochemical decomposition of lignin, on particulate phosphonium-based ionic liquid stationary phases [10]. These stationary phases showed different selectivity compared to reversed-phase columns, even though their hydrophobicity and surface coverage were comparable to C8 silica stationary phases. This confirms that phosphonium-based ionic liquids offer a high

potential as stationary phases for LC separations due to their structure variability, which suggests them to have a number of specific interactions with analytes. However, further research in this area, including a deeper study of the behaviour of phosphonium-based ionic liquids as stationary phases, is needed to get a better insight into the topic.

On the other hand, the first utilization of a nitrogen-based ionic liquid stationary phase for HPLC can be dated back to 2004 [11]. In that work, silica particles were modified by *N,N*-dialkylimidazolium-based salts by a 3-mercaptopropylsilane linker. The prepared stationary phase was applied to the separation of ephedrines under reversed-phase LC (RPLC) conditions. This work opened space for new applications of nitrogen-based salts in separation techniques. Only eight years later, Pino and Afonso published a review article discussing the preparation and LC characterization of more than 20 nitrogen-based ionic liquid stationary phases prepared by modification of spherical silica particles [12]. The preparation of monolithic stationary phases utilizing ionic liquids as precursors for their synthesis is more recent, and silica particles modified by imidazolium-, glucaminium-, or pyrridinium-based ionic liquids still prevail as stationary phases over monolithic stationary phases in LC [13, 14].

The nucleobases and their derivatives are very important nutrients and potential markers useful for therapeutic targets [15, 16]. Their separation is still a challenge due to their high polarity and similarity of structure. A small variation in the chemical structure of such molecules can cause a dramatic difference in their function in living organisms, *e.g.*, methylation as common post-transcriptional modification in DNA and RNA, where an unbalanced ratio between methylation/demethylation processes can lead to various human diseases [17]. These days, reversed-phase and hydrophilic interaction liquid chromatography (HILIC)-tandem mass spectrometry (MS) are the preferred techniques for the analysis of highly complex samples, as confirmed by the number of practical applications gradually

increasing over time [18-22]. The very similar structure of compounds present in real samples complicates their identification and quantification because of co-elution in LC, as well as mutual ion suppression in MS [23]. Thus, stationary phases offering distinct selectivity to this class of compounds can solve such issues at least to some extent.

This study presents the preparation of a new monolithic trioctyl(3/4-vinylbenzyl)phosphonium-based stationary phase and its evaluation under HILIC-UV separation conditions with purine bases, pyrimidine bases, and nucleosides as model compounds.

2 Experimental section

2.1 Chemicals and reagents

Ammonium acetate, acetic acid, acetonitrile (ACN, LC/MS-grade), [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide, styrene, toluene, absolute ethanol, methanol, sodium hydroxide, hydrochloric acid (p.a.), azobisisobutyronitrile (AIBN), xylene mixture of isomers (Product #: 95690), 3-trimethoxysilylpropyl methacrylate (*γ*-maps), and all standard compounds used were purchased from Sigma-Aldrich (Austria). Water purified with a Milli-Q A10 Gradient (Millipore, MA) was used in the experiments. Trioctylphosphine (TOP, 97 %) was purchased from ABCR (Germany) and used without further purification. Vinylbenzyl chloride was purchased from Sigma-Aldrich (Finland), as a roughly 50:50 mixture of the 3 and 4 isomers (Product #: 338729) and used without further purification. Note: Due to high toxicity, vinylbenzyl chloride was kept in the fume hood at all times.

2.2 Synthesis of the phosphonium monomer trioctyl(3/4-vinylbenzyl)phosphonium chloride ([P_{888VBn}]Cl)

The synthesis scheme is shown in **Fig. 1**. TOP (2 mL, 1.66 g, 4.48 mmol) was added to toluene (4.04 g, 4.35 mL, purged with argon for 30 min) in a glass pressure tube, under a flow of argon. Vinylbenzyl chloride (0.684 g, 4.48 mmol) was then added in one portion under argon. The tube was sealed with an argon atmosphere and wrapped in tinfoil. The mixture was left for 64 hours at 70 °C. The reaction mixture was followed by ^{1}H NMR for complete conversion to the phosphonium salt, ensuring no air was allowed to enter the system during sampling. Note: while the presence of oxygen potentially prevents polymerization to some degree, air was kept out of the system to prevent oxidation of the TOP. In general, trialkylphosphines react rapidly with oxygen in the air to give phosphine oxides, but the dissolved oxygen in toluene is quite minimal. It is more essential to keep the reaction sealed so no oxygen is taken up from the air, hence the use of a pressure tube (Ace Glass Pressure Tube, purchased from Sigma-Aldrich, Finland).

The product at the final composition 0.37 g/g in reaction media (toluene) was stored in the fridge, under argon, and wrapped in tinfoil, to avoid polymerization upon concentration and contact with the environment. This regent was used directly for the column preparation.

2.3 Characterization of [P_{888VBn}]Cl by NMR

A small sample of [P_{888VBn}]Cl in toluene was concentrated for NMR analysis, although, full removal of toluene under vacuum (2 mbar) was difficult over a short period (1 hour) as the mixture became very glass-like upon concentration. Further heating of this mixture was not performed, to avoid polymerization of the product. While there was residual toluene in the sample, assignments for the structure were made using a combination of ^{1}H, ^{13}C, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC NMR. Spectra are presented in the Supporting Information (**Figs. S1-S4)**.

The ^{1}H and ^{13}C NMR characterization is as follows: ^{1}H NMR: δ_H (600 MHz, $CDCl_3$) 0.87 (9H, t, *J* 7.0 Hz, $PCH_2(CH_2)_6CH_3$), 1.20-1.30 (24H, m, $PCH_2CH_2CH_2(CH_2)_4CH_3$), 1.36-1.48 (12H, m, $PCH_2CH_2CH_2$ $(CH_2)_4CH_3$), 2.41 (6H, m, $PCH_2(CH_2)_6CH_3$), 4.36 (2H, m, PCH_2Ar), 5.29 (1H, m, ArCH=C*H*H), 5.77 (1H, m, ArCH=CH*H*), 6.68 (1H, m, ArC*H*=CHH), 7.29-7.48 (m, 4H, Ar*H*).

^{13}C NMR: δ_C (150.9 MHz, $CDCl_3$); 14.04 (s, CH_3), 19.89 (d, *J* 46.4 Hz, $PCH_2(CH_2)_6CH_3$), 21.88 (d, *J* 4.8 Hz, $PCH_2CH_2CH_2(CH_2)_4CH_3$), 22.58 (s, $PCH_2CH_2CH_2CH_2(CH_2)_3CH_3$), 27.05 (2*d, *J* 45.6 & 45.8 Hz, PCH_2Ar for the 3 & 4 isomers) 28.91 (d, *J* 2.0, $PCH_2CH_2CH_2CH_2(CH_2)_3CH_3$), 29.00 (s, $PCH_2CH_2CH_2CH_2(CH_2)_3CH_3$), 30.86 (d, *J* 14.6, $PCH_2CH_2(CH_2)_5CH_3$), 31.67 (s, $PCH_2CH_2CH_2CH_2(CH_2)_3CH_3$), 114.88 (s, ArCH=CHH), 115.25 (s, ArCH=CHH), 125.98 (d, *J* 3.6 Hz), 127.06 (d, *J* 3.1 Hz), 127.93 (d, *J* 5.3 Hz), 128.27 (d, *J* 9.8 Hz, Ar), 129.27 (d, *J* 9.0 Hz, Ar), 129.57 (d, *J* 1.5 Hz, Ar), 130.47 (d, *J* 5.3 Hz, Ar), 135.91 (s, ArCH=CHH), 137.65 (s, Ar), 138.76 (s, Ar).

2.4 Instrumentation

The equipment used for HPLC-UV consisted of a syringe pump (100 DM with D-series controller, Teledyne Isco, Lincoln, NE, USA), an electrically actuated E90-220 injection valve with a 60 nL inner loop (Valco, Houston, TX, USA), and a T-splitter with a restrictor (fused silica capillary 0.025 mm i.d. × 150 mm length). The monolithic column outlet was connected to a Spectra 100 UV–Vis detector (Thermo Separation Products, Waltham, MA, USA) via a fused silica capillary of dimensions 0.035 mm i.d. × 110 mm with a bubble cell optical window (0.110 mm i.d.) made by controlled etching. UV-detection was performed at 210 nm. Data were collected and processed using DataApex Clarity 5.02 software.

2.5 Preparation of monolithic capillary columns

Monolithic silica-based capillary columns were prepared using a modified protocol outlined in our previous study [24]. First, 0.88 g of polyethylene glycol (molar mass of 10,000) and 0.9 g of urea were dissolved in 10 mL of 0.01 M acetic acid solution. 2.5 mL of this solution was mixed with 1 mL of tetramethoxysilane and stirred with a magnetic stirrer in a glass vial for 10 minutes. Then, the homogeneous solution was degassed by vacuum in an ultrasonic bath. The fused silica capillary (pre-treated subsequently with 1 M sodium hydroxide at 80°C for 1 hour, water, 1 M hydrochloric acid for 1 hour, water, and nitrogen at 150°C for 3 hours) was filled with the prepared solution and sealed by flame. The reaction was performed at 45°C for 16 hours in a thermostatic environment. Subsequently, the ends of the capillary were cut off and the column was rinsed with water and ethanol (10-20 µL, each). The column was left to dry for 2 days at 25°C and then calcined in a thermostat utilizing a linear temperature gradient from 40°C to 120°C (gradient of 0.5°C/min), and further to 320°C (gradient of 1.0°C/min), followed by a final isothermal step for 2–3 hours. After cooling to ambient temperature, the monolith was modified with 20% (v/v) of *γ*-maps in 95% (v/v) ethanol containing 5% (v/v) of acetic acid and water (1:1). The reaction proceeded at 25°C overnight. The column was flushed with absolute ethanol, dried under a stream of nitrogen at 25°C for 6 hours, and then used for further modification to the appropriate stationary phases.

The zwitterionic monolithic capillary columns were prepared according literature [24]. The phenyl-stationary phase was obtained in a similar way where a solution containing 66 µL of styrene, 300 µL methanol, 700 µL mixture of xylenes, and 1.5 mg of AIBN was used for modification of the silica monolith. The new phosphoniumIL-based stationary phase was prepared using a mixture of 75 mg of [P_{888VBn}]Cl and 2.8 mg of AIBN, dissolved in a mixture of 90 µL methanol and 210 µL toluene. The mixture was degassed in ultrasonic bath under vacuum and pushed through the monolithic column at a flow rate of 8 µL/hour for 16 hours at 80°C. Then the column was flushed with methanol, shortened to 150 mm, and used for HILIC

measurements. The prepared column was extensively flushed with 50/50% (v/v) ACN/100 mmol/L ammonium acetate (pH 4.5) to exchange the chloride ions to acetate ions before HILIC measurements.

3 Results and discussion

The phosphonium monomer [P_{888VBn}]Cl belonging to the group of phosphonium-based ionic liquids was synthesized solely for this study. Its cationic part was immobilized to a silica monolith via *γ*-maps. First, the porosity, permeability, reproducibility of preparation, and stability of the prepared capillary columns were evaluated because these are critical parameters for any chromatographic column from a practical point of view.

3.1 Porosity and permeability of the prepared columns

The total porosity of the prepared monolithic columns reached a value of 0.852 and the permeability was 1.337 x 10^{-14} m^2, calculated as an average for three columns using 80/20% (v/v) ACN/water as the mobile phase. The detailed description of the determination of the total porosity as well as the permeability is presented in our previously published work and the obtained values are comparable to those for zwitterionic sulfobetaine monolithic columns [24]. An increasing concentration of aqueous phase in the mobile phase as well as the addition of acid or ammonium salt to the mobile phase, did not affect these values significantly. This confirms that the grafted layer of polymer does not swell under the applied conditions.

3.2 Reproducibility and stability of the prepared columns

In brief, the data for three monolithic columns prepared in one batch using the same starting mixture containing tetramethoxysilane were used to evaluate the column-to-column reproducibility. The values of the relative standard deviation (RSD) of the retention times for

batch-to-batch reproducibility were calculated for three columns selected from different batches. The column-to-column and batch-to-batch reproducibility of the preparation process for synthesis of silica-based monoliths by acidic hydrolysis of TMOS in the presence of PEG and urea has been discussed before [24]. The lower values of RSDs than for sulfoalkylbetaine modified monoliths were obtained. The RSD of the retention times for the tested analytes did not exceed 4% for column-to-column reproducibility or 5% for batch-to-batch reproducibility, see Supporting Information (**Fig. S-5)**.

The run-to-run repeatability was determined for five consecutive runs using 95/5% (v/v) ACN/100 mM ammonium acetate (pH 4.5) as the mobile phase. The RSD of the retention times for the tested analytes was less than 1%, see Supporting Information (**Fig. S-5)**. This value did not increase to more than 3% after ca. 300 runs. This confirms the stability of the prepared columns under the separation conditions used.

3.3 Separation efficiency of the prepared monolithic columns

The separation efficiency of toluene was comparable on all prepared and characterized columns, *e.g.*, the number of theoretical plates per meter was 137 400 on the phosphonium ligand modified column (**Fig. 2A**) and 123 900 on the bare silica capillary column (**Fig. 2B**), see Supporting Information **Table S-1**. Other compounds differed according to their structure and charge. For example, using the phosphoniumIL-based column (**Fig. 2A**) the efficiency of xanthine reached 174 000 theoretical plates/m, while the tailing peak of partially charged adenine resulted in an efficiency of only 98 300 theoretical plates/meter.

3.4 Comparison of the phosphoniumIL-based stationary phase with other stationary phases

This comparison was done to evaluate the prepared phosphoniumIL-based stationary phase and to screen the effect of present silanol and phenyl groups on the retention of purine and pyrimidine bases and nucleosides under HILIC conditions.

Fig. 2A shows HILIC separation of a sample mixture consisting of toluene as a hold-up volume marker (t_0) and 12 purine and pyrimidine bases and nucleosides, obtained on a phosphoniumIL-based monolithic capillary column with 90/10% (v/v) ACN/100 mmol/L ammonium acetate (pH = 4.5) as the mobile phase. The chemical structures of selected compounds are presented in the Supporting Information (**Fig. S-6)**. The layer of immobilized phosphonium ions on the silica monolith significantly improved the retention and selectivity of the compounds of interest, compared to an unmodified pure silica monolithic column (**Fig. 2B**), and to a monolithic column modified by styrene monomer bonded via *γ*-maps (**Fig. 2C**). The degree of separation and the elution order of the compounds differed significantly on each column.

The bare silica monolithic column (**Fig. 2B**) and phenyl-type column (**Fig. 2C**) showed similar total time of analysis; however, better separation of the selected compounds was obtained on the bare silica monolithic column. The phenyl-type column (**Fig. 2C**) showed the lowest retention for the tested compounds, with partial separation in less than 3.3 minutes. The elution order is mainly governed by the presence of phenyl groups on the stationary phase surface. HILIC separation can be achieved on this type of column because the remaining silanol groups on the monolithic surface enable the creation of a water-rich layer, indispensable for HILIC separation. To demonstrate the unique separation selectivity of the phosphoniumIL-based stationary phase, the column was further compared with a zwitterionic sulfobetaine-stationary phase column (**Fig. 2D**). For example, the retention factor of cytidine (k = 3.394, **Fig. 2D**) is three times higher on the zwitterionic sulfobetaine-stationary phase than on the phosphoniumIL-stationary phase (k = 1.136, **Fig. 2A**) under the selected separation conditions. On the other hand, the retention factor of hypoxanthine (the last eluting compound, 12) was only slightly higher on the phosphoniumIL-based stationary phase (k = 1.590) than on the sulfobetaine stationary phase (k = 1.036). The differences in the selectivity

238 between these columns indicate that each stationary phase offers specific interaction sites
239 contributing to the separation process.

240 *3.5 Hydroxy and methylene group selectivity of the prepared stationary phase*

241 As mentioned before, nucleobases and their derivatives are abundant in the nature and
242 their structures are very similar. Their accurate identification and quantitation is of great
243 interest in metabolomic studies, *e.g*., modified nucleosides are associated with many types of
244 cancer [25-27]. The separation of these compounds is crucial because they usually differ in
245 their molecular structure by only one functional group, *e.g*., a methylene or hydroxyl group.
246 The hydroxy and methylene group selectivity expressed as a ratio between the retention factor
247 of *k*(uridine)/*k*(2-deoxyuridine) at that of *k*(uridine)/*k*(5-methyluridine) can also be used to
248 evaluate the extent of hydrophilic and hydrophobic interactions in different HILIC stationary
249 phases, as suggested by Kawachi *et al*. [28]. In our case, the hydroxy group selectivity
250 (α(OH)) and the methylene group selectivity ($\alpha(CH_2)$) reached values of α(OH) = 2.154 and
251 $\alpha(CH_2)$ = 1.070 for the phosphoniumIL-based stationary phase, while the bare silica monolith
252 gave the same values of α(OH) = $\alpha(CH_2)$ = 1.0159. The sulfobetaine monolithic column
253 showed a comparable hydroxy group selectivity (α(OH) = 2.326), but the methylene
254 selectivity $\alpha(CH_2)$ = 1.754 was almost twice as high as that observed on the phosphoniumIL-
255 based stationary phase.

256 *3.6 Retention and selectivity of the prepared stationary phase*

257 Separation mechanisms on newly synthesized stationary phases are usually evaluated
258 by measuring the effect of the composition of the mobile phase on the separation. The main
259 factor affecting HILIC separation is the ratio between the organic and aqueous part of the
260 mobile phase; however, the presence of acid or buffer in the mobile phase and its pH value
261 also influence the retention and resolution of individual compounds.

The structure of [P_{888VBn}]Cl, used for preparing the capillary monolithic column in the present study, can offer specific interactions with compounds. While the phosphonium cation creates a water-rich layer on the stationary phase surface and supports HILIC interactions, hydrophobic interactions originate from the three octyl chains linked to it. This assumption was confirmed by studying the effect of the concentration of ACN on the retention of toluene and uracil. Toluene is a widely used t_0 marker under HILIC conditions and uracil is a typical t_0 marker under RPLC conditions. The concentration of ACN in the mobile phase was gradually decreased from 95 to 70% (v/v), the ammonium acetate buffer pH was 4.5, and its concentration in mobile phase was kept at 5 mmol/L. The retention factor (k) for uracil decreased with increasing concentration of aqueous phase in the mobile phase, and was 0.720 with 95% ACN, 0.359 with 90% ACN, and 0.115 with 80% ACN in the mobile phase. The reversed-phase separation mechanism became dominant when the mobile phase contained 30% (v/v) of buffer; see the elution order of toluene and uracil in **Fig. 3**.

3.7 Effect of additive in the mobile phase on HILIC separation of purine and pyrimidine bases and nucleosides

To evaluate the effect of the presence of buffer or acid in the mobile phase as well as the effect of pH value, a mobile phase containing 95% (v/v) of ACN was chosen due to bigger differences in the retention factors of the selected compounds. **Table 1** summarizes the retention factors k for the selected compounds obtained on the prepared column using a mobile phase containing 95% of ACN and 5% of water, acetic acid, or ammonium acetate.

Comparing the results presented in **Table 1**, one can see that the retention factors for the ACN/water system were almost identical to those using 95/5% (v/v) ACN/100 mmol/L ammonium acetate at pH 4.5. In general, we observed that a lower concentration (0.5 mmol/L) of acetic acid or ammonium acetate in the mobile phase resulted in higher retention

286 factors of the compounds than using pure water or higher concentrations of additives in the
287 mobile phase. Later eluting compounds were more affected than early eluting compounds.
288 This trend is independent on the pH value of the mobile phase. If the concentration of
289 additives in the mobile phase was lowered from 5 to 0.5 mmol/L, the retention factors
290 increased. For example, for uridine there was an increase in *k* of about 15% using acetic acid
291 (**Fig. 4A**), a 31% increase using ammonium acetate at pH 4.5 (**Fig. 4B**), and a 17% increase in
292 *k* using ammonium acetate at pH 6.8 (**Fig. 4C**).

293 In contrast to this finding, the sulfobetaine stationary phase behaved in an opposite
294 way; an increasing concentration of ammonium acetate in the mobile phase lead to an
295 increase in the retention of the compounds, except for adenine. The retention of adenine was
296 unaffected by a change in the ammonium acetate concentration from 0.5 to 5 mmol/L in the
297 mobile phase. This data confirms that the concentration of additives in the mobile phase
298 affects not only the creation of a water-rich layer on the stationary phase surface, but also the
299 charge of the compound in the mobile phase, and therefore the contribution of electrostatic
300 interactions to the retention of the compounds.

301 The presence of mobile phase additives also affected the resolution between individual
302 compounds, *e.g.,* thymine and uracil or 2-deoxyuridine and cytosine. Especially for the
303 separation of the latter couple of compounds, a higher concentration of additives in the mobile
304 phase is required. The relevant chromatograms are presented in **Fig. 4**. Although 2-
305 deoxyuridine and cytosine (compounds 4 and 5) were almost baseline separated with an
306 ACN/water mobile phase (**Fig. 5**, bottom chromatogram), adding 0.5 mmol/L of acetic acid or
307 ammonium acetate to the mobile phase resulted in co-elution of these compounds (**Fig. 4**
308 upper figures). However, further increasing the mobile phase ammonium acetate
309 concentration to 5 mmol/L gave partial (**Figs. 4A** and **C** bottom) or full (**Fig. 4B** bottom)
310 baseline resolution of this pair of compounds. Thymine and uracil (compounds 2 and 3)

311 behaved oppositely (see **Fig. 4A-C**). The increase in concentration of ammonium acetate in
312 the mobile phase lowered the resolution between these compounds.

313 *3.8 Equilibration time of the phosphoniumIL-based stationary phase*

314 The conditioning of the column and the equilibration time are essential for good
315 retention time repeatability under HILIC conditions. For example, stationary phases bearing
316 amino groups require equilibration with an appropriate mobile phase for several hours when
317 used for the first time and later on a 3-hour equilibration time is adequate to get repeatable
318 analyses [28]. The prepared phosphoniumIL-based stationary phase exhibited similar
319 behaviour using mobile phases containing pure water and ACN. The separation of a sample
320 mixture containing purine and pyrimidine bases and nucleosides utilizing ACN/pure water
321 mobile phase (95/5% (v/v)) after 1 hour of equilibration showed very high retention for all the
322 compounds (**Fig. 5**, upper chromatogram). The peak shapes of most of the compounds were
323 poor, and hypoxanthine (compound no. 12) was not even detected due to its broad peak.
324 However, increasing the equilibration time markedly improved the peak shapes and shortened
325 the analysis time. When the columns were flushed with the mobile phase for 4 hours (**Fig. 5**,
326 middle chromatogram), the separation was significantly shortened to 18 minutes (compared to
327 40 minutes using only 1 hour of equilibration), and the resolution for early eluting compounds
328 such as thymine and uracil (compounds numbered 2 and 3) was improved from 1.04 to 4.37.

329 These results show that separations using mobile phases containing ACN and pure
330 water require equilibration times longer than 8 hours to create a stable water-rich layer on the
331 stationary phase surface and to get repeatable elution times under HILIC conditions (**Fig. 5**
332 (bottom)). It should be noted, however, that our synthesized stationary phase behaved in this
333 way only in the presence of pure water in the mobile phase. Even a low concentration of

334 acetic acid or ammonium acetate in the mobile phase, *i.e.*, 0.5 mmol/L, shortened the
335 equilibration time to 1 hour, which corresponds to 28 exchanged column volumes.

336 **4 Conclusions**

337 The presented results confirm that phosphonium-based ionic liquids can be
338 successfully used for the preparation of monolithic stationary phases, suitable for HILIC. The
339 novel silica-based monolithic capillary column modified by [P_{888VBn}]Cl presented in this work
340 has some distinct advantages, such as, short equilibration time in mobile phases containing
341 acids or salts (less than 1 hour), high efficiency originating from the structure of the silica
342 monolith (over 100 000 theoretical plates/m), and high stability under HILIC conditions (can
343 withstand more than 300 analyses). The phosphonium-based stationary phase offers better
344 selectivity to compounds of interest than silica, phenyl, and sulfobetaine monolithic columns
345 evaluated in this study. Our results show that such stationary phase is a good alternative for
346 analyses where fast and efficient separation of nucleobases and their derivatives is required.
347 This opens the way to utilize such stationary phase in research fields, such as, proteomics,
348 metabolomics, or foodomics, where highly complex samples are processed.

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Figure caption:

Figure 1. The scheme of synthesis of trioctyl(3/4-vinylbenzyl)phosphonium chloride ([P_{888VBn}]Cl).

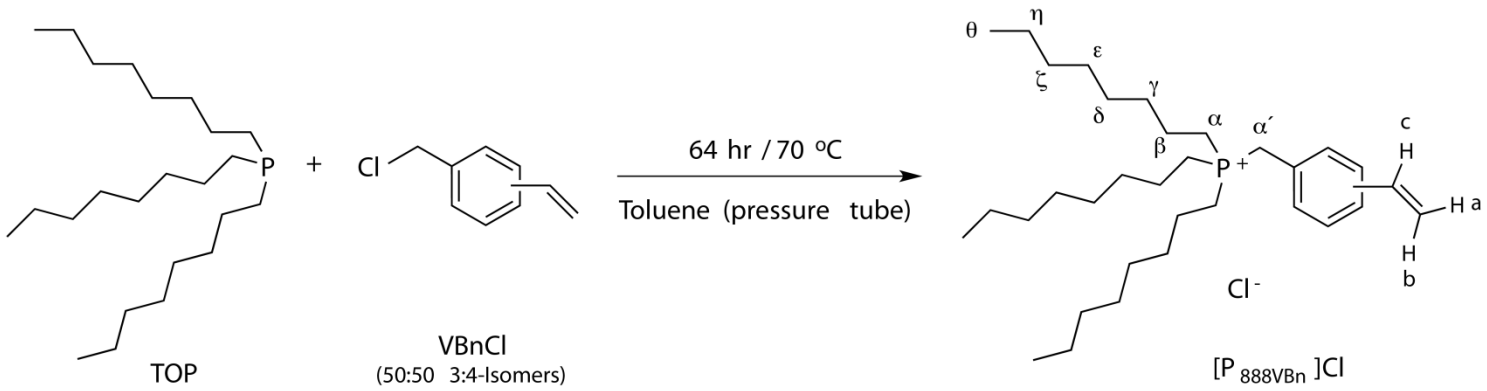
Figure 2. HILIC separation of purine and pyrimidine bases and nucleosides.

(A) PhosphoniumIL-based stationary phase; (B) Bare silica monolith; (C) Phenyl-stationary phase; (D) Zwitterionic sulfobetaine-stationary phase. Mobile phase: 90/10% (v/v) ACN/100 mmo/L ammonium acetate at pH 4.5; capillary column 150 x 0.1 mm; flow rate 500 nL/min; UV-detection at 210 nm. Peak identification: toluene (t_0 marker), (1) xanthine, (2) thymine, (3) uracil, (4) 2-deoxyuridine, (5) cytosine, (6) 2-deoxycytidine, (7) adenosine, (8) 5-methyluridine, (9) uridine, (10) cytidine, (11) adenine, (12) hypoxanthine.

Figure 3. Reversed-phase mechanism on the synthesized phosphoniumIL-based stationary phase. Mobile phase: 70/30% (v/v) ACN/ammonium acetate buffer at pH 4.5, 5 mmol/L in mobile phase. All other conditions as in **Fig. 2**.

463 **Figure 4.** The effect of additives to the mobile phase on the HILIC separation of purine and
464 pyrimidine bases and nucleosides. Mobile phase: 95% ACN + (A) 5% acetic acid, (B) 5%
465 ammonium acetate pH 4.5, or (C) 5% ammonium acetate at pH 6.8. Other conditions and
466 peak identification as in **Fig. 2**.

467 **Figure 5.** Equilibration of phosphoniumIL-based stationary phase in the ACN/water mobile
468 phase. Mobile phase: 95/5% (v/v) ACN/water. Other conditions and peak identification as in
469 **Fig. 2**.



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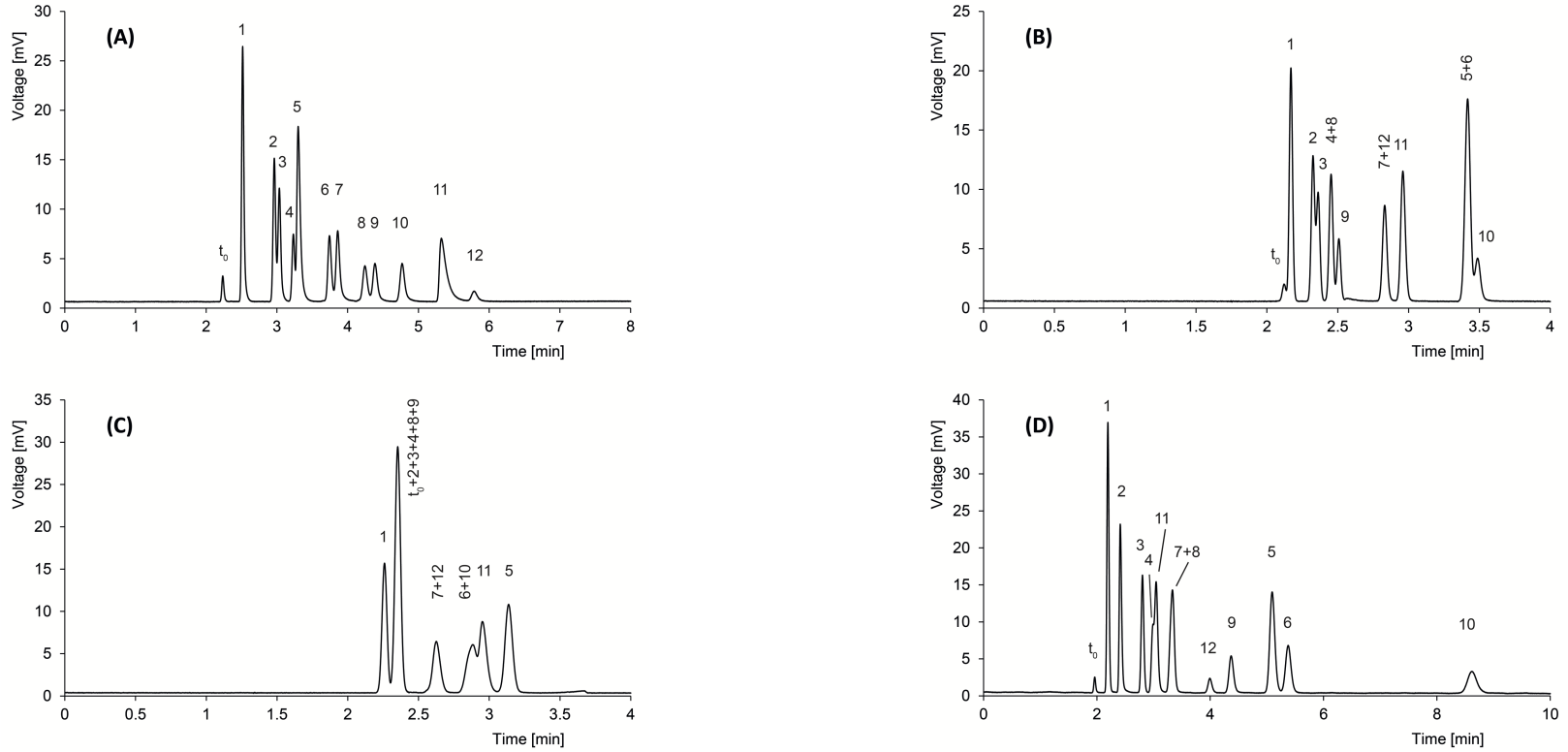

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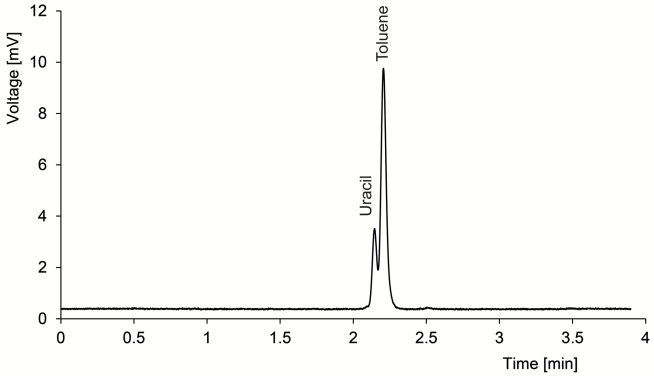

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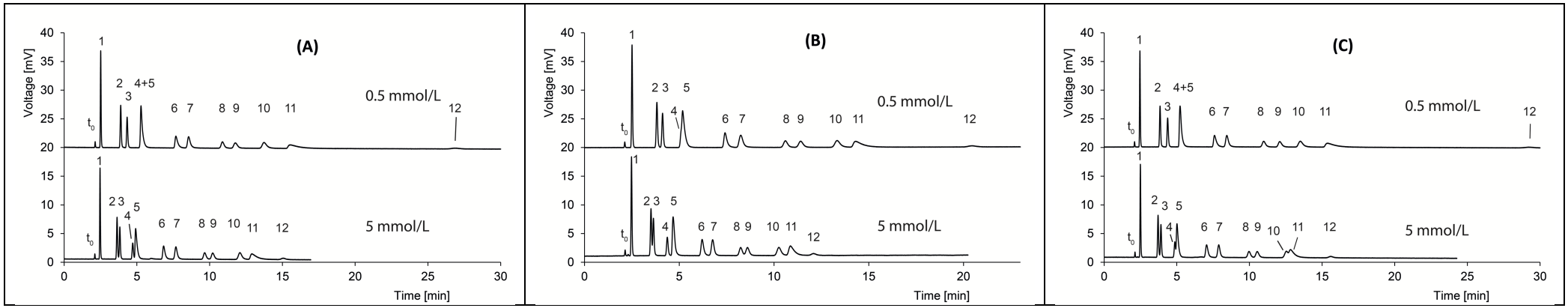

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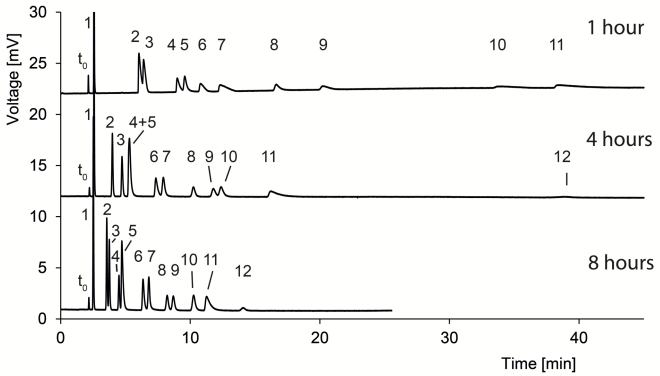

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